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Analysis of multiple pesticide residues in tobacco using pressurized liquid extraction, automated solid-phase extraction clean-up and gas chromatography–tandem mass spectrometry

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Abstract

A new method was developed for the analysis of pesticide residues in tobacco. The objective was to significantly increase the number of samples that can be processed by the laboratory and to enable the extension of the current coverage to additional pesticides. A new analytical approach was therefore defined based on two main axes, the automation of the sample preparation and the selectivity of the analyte detection using tandem mass spectrometry. This latter aspect reduces the stringency of the requirements placed on the clean-up of the extracts and on the chromatographic resolution when less selective detectors are used. The extraction of the analytes from the matrix is performed using the pressurized liquid extraction technique. Tobacco samples are extracted at elevated temperature and pressure (100 °C and 100 atm; 1 atm = 101,325 Pa) using acetone as an extraction solvent. The resulting extract is then concentrated using a Vortex evaporator. Three different solid-phase extraction (SPE) procedures, adjusted to the chemical properties of the different active ingredients to be measured, are applied to the concentrated extract, thus leading to three extract fractions. The first fraction contains such main classes of active ingredients as organohalogenated and 2,6-dinitroaniline compounds while the second one collects the organophosphorus and acylalanines residues; these two fractions are analyzed by capillary gas chromatography coupled to tandem mass spectrometry using negative chemical ionization and electron impact ionization in the positive mode, respectively. The third extract fraction gathers the *N*-methylcarbamates residues which are analyzed by HPLC with post-column derivatization and fluorescence detection. The different sample preparation stages from extraction to SPE clean-up have been automated through the use of recent analytical technologies. In combination with the analysis by tandem mass spectrometry, this provided a potential for a high sample throughput.
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1. Introduction

Public concern over residues of pesticides in food and related commodities has been increasing during the past 20–25 years. This situation has led to regulations setting maximum residue limits (MRLs) [1] of pesticide residues in different agricultural commodities including tobacco and/or tobacco products. Laboratories are thus requested to analyze many samples for a wide range of active ingredients. The latter belong to different chemical classes, such as organochlorine and organophosphorus compounds as well as *N*-methylcarbamates and dinitroanilines

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which require different analysis strategies. As a consequence, a particular difficulty when developing methods for residue analysis is the need to cover a wide range of different chemicals, using one single or a minimum of specific procedures.

In order to cover the main part of the active ingredients monitored by our laboratory, the procedures currently applied include the ISO Standard 4389 [2] that covers 17 chlorinated compounds and a multiresidue method very close to method S-19 of the Manual of Pesticide Residue Analysis [3] of the Deutsche Forschungsgemeinshaft of which one mission is to advise public authorities on scientific questions. This multiresidue procedure, derived from the S-19 method, is the core of the methodology applied in our laboratory. It covers 44 active ingredients including mainly organophosphorus, *N*-methylcarbamates and chlorinated compounds which cannot be analyzed by the ISO 4389 Standard. The basic analysis strategy applied involves solvent extraction, clean-up including partitioning, gel permeation chromatography and column chromatography for additional fractionation. The pesticides are then determined by GC and HPLC using various column/detector combinations to achieve the necessary selectivity and sensitivity for the different classes of compounds. The samples which test positive for targeted pesticides are reanalyzed using a second (confirmation) column of different polarity.

Altogether, the presently used method is very labor-intensive and provides a limited sample capacity. It appeared therefore necessary to have a method which would allow the processing of more samples, without an increase in labor. This is the reason why a new multiresidue method was developed, incorporating recent instrumental techniques, such as pressurized liquid extraction (PLE; Dionex trade name ASE for accelerated solvent extraction), automated solid-phase extraction (SPE) and tandem mass spectrometry (MS–MS). Time consuming stages of the current method could thus be replaced by automated sample processing operations that significantly reduced the workload required for the analysis.

1.1. Pressurized liquid extraction (PLE)

PLE is an automated extraction technique where solid or semi-solid samples are extracted with liquid solvents at elevated temperatures (50–200 °C) and pressures (5–200 atm; 1 atm = 101,325 Pa). PLE is being increasingly used for the extraction of pesticides from different matrices [4–6]. This technique meets the requirements of the US Environmental Protection Agency (EPA) for the extraction, from soils and waste solids, of a vast number of classes of organic compounds, including organochlorine and organophosphorus insecticides as well as chlorinated herbicides [7]. Due to the elevated pressure and temperature applied in PLE, the extraction times are shorter (<20 min) and it is possible to use organic solvents of lower polarity than it would be required when applying conventional methods (e.g. pure acetone instead of acetone–water or acetonitrile–water mixtures). This results in cleaner extracts which can be directly submitted to SPE as opposed to the extracts obtained with aqueous solvent systems which require filtration or partition prior to the SPE treatment.

PLE also presents advantages compared to other recent extraction techniques such as supercritical fluid extraction (SFE) and microwave-assisted extraction (MAE). Camel [8] reported that the main advantages of SFE are the possibility of performing the extraction of thermolabile compounds at moderate temperature (around 40 °C) and that of achieving a high level of selectivity when CO_2 is used as the extractant. However, an important drawback of SFE is the difficulty of extracting polar compounds. This requires the addition of an appropriate modifier to the fluid, leading to very tedious method optimization stages. Instead, a wide range of different extractants can be used for PLE and MAE and therefore these techniques can be successfully applied to all types of solutes and of solid matrices. In addition, among the commercially available systems, PLE instruments are more automated than MAE systems. The latter require manual operations such as loading the solvent into the cell and a filtration or centrifugation stage to separate the extract from the matrix at the end of the extraction process. The applicability of the technique to a wide range of different analytes, the compatibility of the extracts, as obtained, with the subsequent clean-up stages and the commercial availability of automated systems were determinant factors for the selection of PLE for the present multiresidue method.

1.2. Automated solid-phase extraction and clean-up

Various SPE automated systems are now commercially available and allow to handle complex experimental conditions, requiring, e.g. several elution solutions and sequential SPE procedures using different stationary phases. Some instruments using SPE well plates are designed to handle batches of up to 96 samples and thus provide a capability for high sample throughput.

1.3. Tandem mass spectrometry (MS–MS)

MS–MS is still an emerging technique in the field of residue analysis [9–13] and represents an attractive alternative to element-specific detection and to standard MS. Compared to the use of element-specific detectors, the detection by mass spectrometry offers two major advantages: (1) the substitution of an array of element-specific detectors by a single detector based on *m*/*z*; (2) the unambiguous detection of residues of active ingredients according to their mass spectral characteristics. However, for the analysis of residues in tobacco samples, standard MS instruments proved not selective enough for the analysis of a large number of active ingredients in the frame of a multi-residue method. The spectral information obtained using such instruments was very much limited by interferences from the matrix ions and by an elevated chemical noise decreasing the sensitivity of the detection.

MS–MS provides an additional level of filtering that allows to measure unequivocal mass spectral characteristics of the analytes. This leads to a very important increase of the selectivity and of the signal/noise ratio compared to standard instruments. Thus, MS–MS offers, particularly in the case of complex matrices, lower limits of detection (LOD) and more reliable identifications of the detected residues. In addition, the high selectivity achieved allows an imperfect chromatographic separation, a fact that can be exploited to drastically shorten the analysis time: The sample preparation and clean-up can be simplified and often a full separation of chromatographic signals is unnecessary. The two types of tandem mass spectrometers most frequently employed for trace analyses in complex matrices are triple quadrupole and ion trap instruments. Only few direct comparisons of these two instrumental configurations exist for trace analyses [14]; however, it is generally recognized that triple quadrupole mass spectrometers operated in the selected reaction monitoring (SRM) mode, offer a higher sensitivity and, in particular, better residual standard deviations for quantitation experiments than ion trap instruments.

A general flow-chart of the new method is shown in Fig. 1. A tobacco extract prepared by PLE is first concentrated and cleaned up by three different SPE procedures according to the chemical properties of the active ingredients to be measured. Three extract fractions are obtained: Extract fraction 1 contains the organohalogenated and 2,6-dinitroaniline compounds and is analyzed by GC–MS–MS in the negative-ion mode. Extract fraction 2, containing the acylalanines and organophosphorus pesticides, is analyzed by GC–MS–MS in the positive-ion mode. Finally, extract fraction 3 containing *N*-methylcarbamates is analyzed by HPLC using fluorescence detection.

2. Experimental

2.1. Reagents and material

Acetone, isooctane and toluene (pesticide grade), were obtained from Fluka (CH-9471 Buchs, Switzerland). With three exceptions, all pesticide standards were purchased from Dr. Ehrenstorfer Co. (Augsburg, Germany). Terbufos sulfone was purchased from Supelco (Buchs, Switzerland), deuterium-labeled ethylene dibromide (EDB) was obtained from Cambridge Isotope Labs. (Andover, MA, USA) and camphechlor was purchased from Promochem (Wesel, Germany). One milliliter SPE cartridges containing 200 mg of adsorbent (Florisil, silica gel, ENVI-Carb, aminopropyl) were obtained from Supelco. Hydromatrix (diatomaceous earth), was purchased from Varian (Basel, Switzerland). The reagents for *N*-methylcarbamate analyses [thiofluor, *o*-phtal-aldehyde (OPA) and OPA diluent] were obtained from Pickering Labs. (Mountain View, CA, USA).

2.2. Sample extraction

Pressurized liquid extraction

Extraction was carried out using an ASE 200 instrument (Dionex, Sunnyvale, CA, USA). This instrument features an auto-sampler carousel and a

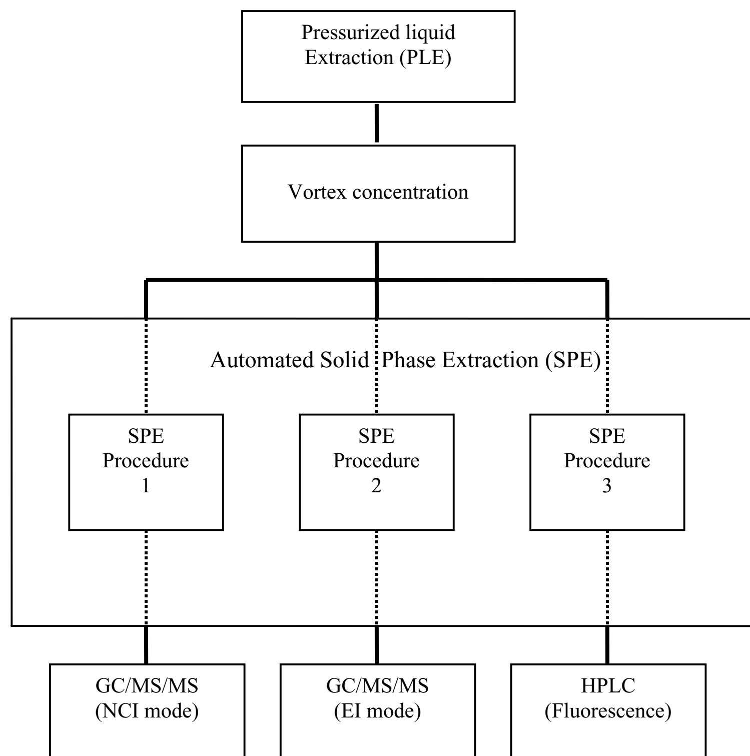

Fig. 1. Schematic illustration of the method.

collection tray allowing the unattended extraction of up to 24 samples. Stainless steel extraction cells and glass collecting vials with 33 and 40 ml volumes, respectively, were used.

Ground tobacco (7.5 g) having a moisture content of approximately 5% was mixed with 3 g of hydromatrix. The mixture was filled into the extraction cell. Then, 0.5 ml of internal standard solution (75 μg/ml of thiofanox, 60 μg/ml of mirex and 15 μg/ml of [$^2H_4$]-EDB in acetone) was added. The free cell space was filled up with Hydromatrix. The cell was then closed and placed on the PLE vial tray. Acetone was used as extraction solvent under the following PLE conditions: temperature: 100 °C; pressure: 100 atm; heating time: 5 min; static extraction time: 3 min; flush volume: 60% of extraction cell volume; purge: N_2, 60 s; number of cycles: 3. The total volume of extract obtained under those conditions was 60 ml and it showed only very little variations, less than 3 ml, when analyzing different samples. These differences were compensated by the presence of the internal standard.

2.3. Sample concentration and clean-up

2.3.1. Concentration conditions

Each PLE extract was concentrated to 1 ml by vortex evaporation using a multi-sample TurboVap LV evaporator (Zymark Hopkinton, MA, USA). The concentration was performed in 50 ml round-bottomed flasks. One ml of toluene was added as keeper prior to the concentration. Evaporation then took place according to the following conditions: bath temperature, 55 °C; air pressure, 0.7 atm; evaporation time, 18 min; residual solvent volume, 1 ml. After evaporation, each residue was transferred to a 2 ml vial and the extraction tube was rinsed with two times 0.5 ml of toluene.

2.3.2. Solid-phase extraction (SPE) clean-up

SPE clean-ups were performed using a Gilson 215 SPE automated unit, (Gilson Labs., Villiers-le-Bel, France). To cope with the differences in the chemical properties of the considered active ingredients,

three clean-up procedures were defined. Each procedure consisted of two SPE steps in series. The first clean-up procedure was dedicated to low-polarity compounds, i.e. organochlorine compounds and 2,6-dinitroanilines. A 0.1 ml aliquot of the concentrated extract was loaded onto a Florisil cartridge previously conditioned with 2 ml of toluene (flow rate 3 ml/min). The SPE column was then washed with 0.1 ml of isooctane–toluene (50:50) (flow rate of 3 ml/min). The pesticides were eluted with 0.7 ml of isooctane–toluene (50:50) at a flow rate 4 ml/min and the eluate was collected in a 2.2 ml well of the sample collection rack. The content of the well was automatically transferred onto a second SPE cartridge (silica gel) previously conditioned with 2 ml of toluene (flow rate 5 ml/min). The first 0.1 ml eluting from the SPE column was discarded while the rest was collected. The elution of the pesticides was performed with 1 ml of toluene (flow rate 5 ml/min). The eluate (extract fraction 1; total volume 1.6 ml), was analyzed without further treatment by GC–MS–MS in the negative chemical ionization (NCI) mode.

The second clean-up procedure was designed for compounds of intermediate and/or high polarity, including acylalanines, organophosphorus compounds and some halogen-containing pesticides. A 0.1 ml aliquot of the concentrated extract was loaded onto a silicagel cartridge, previously conditioned with 2 ml of acetone (flow rate 3 ml/min). The SPE column was washed with 0.2 ml of toluene (flow rate 3 ml/min). The pesticides were then eluted with 1.1 ml of a mixture of acetone–toluene (83:17) (flow rate 6 ml/min). The first 0.3 ml of the effluent were discarded while the rest was collected in a 2.2 ml well of the sample collection rack. The content of the well was then automatically transferred onto a second SPE cartridge (graphitized carbon black) previously conditioned with 2 ml of acetone (flow rate 3 ml/min). The elution of the pesticides was performed with 0.8 ml of acetone and the eluate (extract fraction 2; total volume 0.8 ml), was collected and analyzed without further treatment by GC–MS–MS using electron impact (EI) ionization in the positive mode.

The third clean-up procedure was performed in order to purify a fraction of the concentrated extract for the *N*-methylcarbamate analysis. A 0.1 ml aliquot of the concentrated extract was loaded onto an aminopropyl cartridge, previously conditioned with 1 ml of acetone (flow rate 3 ml/min). The column was washed with 0.15 ml of a mixture of methanol–ethyl acetate (50:50) (flow rate 3 ml/min). The pesticides were then eluted with 0.5 ml of a mixture of methanol/ethyl acetate 50:50 (flow rate 3 ml/min) and the column effluent was collected in a 2.2 ml well of the sample collection rack. The content of the well was automatically transferred onto a second SPE cartridge (graphitized carbon black) previously conditioned with 2 ml of methanol (flow rate 3 ml/min). This column was washed with 0.05 ml of methanol (flow rate 3 ml/min) that was discarded. The *N*-methylcarbamates were then eluted with 0.55 ml of methanol (flow rate 3 ml/min). The resulting eluate (extract fraction 3, total volume 0.5 ml) was analyzed without further treatment by HPLC coupled to a post column derivatization system and to a fluorescence detector.

Six 96-well SPE plates and the corresponding sample collection racks could be loaded on the automated SPE instrument. Thus, all six SPE steps described above could be performed in a totally unattended manner for a typical analysis batch of 24 samples. The instrument was programmed to first perform the clean-up procedure dedicated to the low-polarity compounds, then the procedure for compounds of intermediate or high polarity and, finally, the clean-up procedure for the *N*-methylcarbamates. For each individual SPE step, eight samples were processed in parallel. Under those conditions, the time needed to process 24 samples was approximately 70 min for each of the three clean-up procedures, thus leading to an overall clean-up time of 3.5 h.

2.4. Residue analysis and quantification

Extract fraction 1 containing organochlorine compounds, pyrethroïds and 2,6-dinitro-anilines and extract fraction 2, containing the acylalanines, organophosphorus and certain halogenated pesticides were analyzed by GC–MS–MS; extract fraction 3 containing the *N*-methylcarbamates was analyzed by HPLC with post-column derivatization and fluorescence detection.

2.4.1. GC–MS–MS analysis of extract fraction 1

A HP-6890 gas chromatograph equipped with a split/splitless injector and an auto-sampler from

Agilent Technology (Waldbronn, Germany) was used. The separation was carried out on a HP–5 capillary column, 30 m × 0.25 mm i.d., 0.20 μm film thickness from Agilent Technology equipped with a 3 m pre-column of the same type. One microliter of extract fraction 1 was injected using the pulsed splitless mode (split valve closed for 1 min, helium pressure, 250 kPa, 0.5 min) and an injector temperature of 210 °C. The initial oven temperature was held at 57 °C for 2 min; it was increased first at 40 °C/min to 130 °C, then at 5 °C/min to 200 °C, and finally at 7 °C/min to 270 °C and maintained at that temperature for 10 min. Helium was used as carrier gas in the ramped pressure mode: the pressure initially of 64 kPa was increased at a rate of 3 kPa/min to 160 kPa and maintained at that level for 10 min.

Mass spectrometer: A TSQ-7000 triple quadrupole tandem MS system from Thermo-Finnigan (San Jose, CA, USA) equipped with an EI/CI ion source and X-calibur data treatment system. The NCI mode was employed using methane (4000 mTorr; 1 Torr = 133.322 Pa) as the reagent gas and the electron energy was set at 200 eV.

The acquisition was carried out using the SRM mode with the transitions reported in Table 1. Collision activated decomposition (CAD) was performed using argon as the collision gas at 1 mTorr, the same collision offset of 20 V was applied for all transitions.

The quantitation of the active ingredients was performed by internal standard single point calibration, using a calibration solution containing 0.5 μg/ml of the different active ingredients except for eight compounds, the concentration of which was adapted with the purpose of either avoiding the saturation of the electron-multiplier by compounds having a very high response factor (flumetralin, benfluralin, trifluralin) or optimizing the shape and ease of integration of the chromatographic signals of selected pesticides (β-hexachlorocyclohexane (β-HCH) and four individual cypermethrins). The concentrations of these compounds in the calibration solution were as follows: benfluralin, 0.25 μg/ml; trifluralin, 0.20 μg/ml; flumetralin, 0.25 μg/ml, β-HCH, 1.0 μg/ml and four individual cypermethrins, 0.20–0.30 μg/ml. Mirex was used as internal standard, at a concentration of 4 μg/ml).

Table 1
SRM transitions for the different analytes in the NCI mode

	Active ingredient	Type	t_R (min)	Precursor ion	Product ion
1	Trifluralin	DN	13.10	335	305
2	Benfluralin	DN	13.17	335	305
3	α-HCH	OC	13.44	255	35
4	Hexachlorobenzene (HCB)	OC	13.72	284	35
5	Dichloran	OC	13.87	255	35
6	β-HCH	OC	14.42	255	35
7	Lindane	OC	14.58	255	35
8	δ-HCH	OC	15.47	255	35
9	Heptachlor	OC	17.13	300	35
10	Aldrin	OC	18.33	330	35
11	Butralin	DN	19.29	295	46
12	Isopropalin	DN	19.56	309	279
13	Pendimethaline	DN	19.76	281	46
14	Heptachlor epoxide	OC	19.83	353.6	35
15	*trans*-Chlordane	OC	20.46	410	35
16	*o,p*-DDE	OC	20.68	246	35
17	α-Endosulfan	OC	20.83	406	35
18	Flumetralin	DN	21.12	421	174
19	*p,p*-DDE	OC	21.67	318	35
20	Dieldrin	OC	21.63	380	35
21	*o,p*-DDD	OC	21.90	248	35
22	Nitrofen	OC	22.27	283	138
23	Endrin	OC	22.25	380	35
24	β-Endosulfan	OC	22.54	406	35
25	*p,p*-DDD	OC	22.91	248	35
26	*o,p*-DDT	OC	22.98	246	35
27	Endosulfan_sulfate	OC	23.79	422	97
28	*p,p*-DDT	OC	23.95	318	35
29	λ-Cyhalothrin-1	PY	26.69	449	205
30	λ-Cyhalothrin-2	PY	26.97	449	205
31	*cis*-Permethrin	PY	27.98	207	35
32	*trans*-Permethrin	PY	28.17	207	35
33	α-Cypermethrin	PY	29.46	207	35
34	β-Cypermethrin	PY	29.65	207	35
35	γ-Cypermethrin	PY	29.79	207	35
36	δ-Cypermethrin	PY	29.86	207	35
37	*RS/SR*-Fenvalerate	PY	31.28	211	167
38	*SS/RR*-Fenvalerate	PY	31.78	211	167
39	*cis*-Deltamethrin	PY	32.60	297	81
40	*trans*-Deltamethrin	PY	33.14	297	81
41	Camphechlor	OC	24.81	413	35
42	Mirex (istd)	OC	26.53	368	297

OC, organochlorine compounds; DN, dinitroanilines; PY, pyrethroïds; istd, internal standard.

2.4.2. GC–MS–MS analysis of extract fraction 2, EI ionization in the positive mode

The same instrumentation and chromatographic column as reported above were used. One microliter

Table 2
SRM transitions for the different analytes in the EI mode

	Active ingredient	Type	t_R (min)	Precursor Ion	Product ion
1	EDB	OB	3.11	186	107
2	Dimefox	OP	5.43	154	44
3	DBCP	OC	6.01	157	75
4	Methamidophos	OP	7.59	95	47
5	Dichlorovos	OP	7.68	185	93
6	Mevinphos	OP	9.32	192	127
7	Acephate	OP	9.37	136	42
8	Dimethoate	OP	11.72	143	111
9	Terbufos	OP	12.07	231	175
10	Diazinon	OP	12.22	304	179
11	Disulfoton	OP	12.30	274	88
12	Formothion	OP	12.58	170	93
13	Methyl parathion	OP	12.89	125	47
14	Metalaxyl	AC	13.05	206	132
15	Ethofumesate	OP	13.27	286	207
16	Malathion	OP	13.37	173	99
17	Fenthion	OP	13.50	278	109
18	Ethyl parathion	OP	13.54	291	109
19	Terbufos sulfone	OP	13.97	264	153
20	Disulfoton sulfone	OP	14.41	213	153
21	Fenamiphos	OP	14.56	303	260
22	Benalaxyl	AC	15.63	325	148
23	Methoxychlor	OC	16.42	344	227
24	Phosalone	OP	16.81	367	182
25	$^{2}H_4$-EDB (istd for EDB)	OB	3.08	194	113
26	Mirex (istd)	OC	17.04	272	237

OC, organochlorine compounds; OB, organobromine compounds; AC, acylalanines; istd, internal standard.

of extract fraction 2 was injected using the splitless mode (split valve closed for 1 min) at an injector temperature of 210 °C. The oven temperature was held at 57 °C for 1.5 min, then increased at 15 °C/min to 290 °C where it was maintained for 15 min. Helium was used as carrier gas in the constant flow mode (1.2 ml/min).

EI mass spectra were measured at an ionization energy of 70 eV. The acquisition was carried out in the SRM mode, using the transitions reported in Table 2. CAD was performed using argon as collision gas at 0.13 mTorr; a collision offset of −12 V was applied for all transitions.

The quantitation of the active ingredients was performed by internal standard, single-point calibration using a calibration solution containing 0.5 μg/ml of each of the internal standards, mirex and $^{2}H_4$-EDB, as well as of the different active ingredients except acephate and methamidophos which were present at 0.25 μg/ml.

2.4.3. HPLC analysis (fluorescence detection) of extract fraction 3

A high-performance liquid chromatograph, HPLC model 600 E, equipped with an automatic sampler, model 717 plus, from Waters (Milford, MA, USA) was used. The detection of the *N*-methylcarbamates was achieved using a post-column derivatization module, model PCX 5100 from Pickering Labs. and a scanning fluorescence detector model 474 from Waters.

The separation was carried out using a Pickering C_{18} analytical column for carbamate analysis (250 mm × 3.9 mm i.d., 5 μm packing). A guard column (130 mm × 4.6 mm i.d.) with the same stationary phase was used. The instrument settings were the following: injection volume, 10 μl; HPLC column temperature, 42 °C; derivatization coil temperature, 100 °C; excitation wavelength, 330 nm; emission wavelength, 465 nm.

The mobile phase consisted of two solvents, water (A) and methanol (B). The composition of the mobile phase was varied as follows: The initial composition of 80% solvent A was maintained for a 9 min hold period, then the proportion of solvent A was lowered to 20% at 50 min, according to a linear gradient. At 50 min, the composition of the mobile phase was changed in one step to 100% of solvent B. This composition was maintained for a hold period of 5 min to provide column clean-up. Finally, the solvent composition was brought back to the initial conditions, 80% solvent A, according to a 6 min linear gradient and was maintained for 16 min in these conditions for column conditioning.

The quantitation of the active ingredients was performed by internal standard, single point calibration using a calibration solution containing 0.5 μg/ml of the different *N*-methylcarbamates and 4.0 μg/ml of thiofanox as internal standard.

3. Results and discussion

3.1. Evaluation of the PLE conditions

The PLE conditions given in the EPA method were applied. These conditions which are similar to those used by many authors were recommended by the EPA

[7] for the extraction of a wide range of organic compounds including organochlorine and organophosphorus pesticides from soils and waste solids.

In our case, the recovery of the analytes of interest was determined using tobacco samples fortified with all the active ingredients to be covered by the method. The internal standard, mirex, was added to the sample in the extraction cell. The resulting tobacco extracts were then cleaned up and analyzed according to existing methods. Satisfactory recoveries were obtained (ranging between 75 and 125%). Besides, the extraction was shown to be consistent between runs as the coefficients of variation ranged between 4 and 12% (for 6 replicate analyses at a fortification level of 0.5 ppm).

3.2. Concentration of the extract

The PLE extracts need to be concentrated prior to loading on the SPE cartridges. A Vortex evaporator was used, that allowed to process simultaneously the 24 extracts of the PLE run. The concentration was performed in 50 ml round-bottomed sample tubes. It was observed that, for a given operating temperature, these tubes allowed to achieve the required concentration in a shorter time (by factors up to 2.5) compared to conical flasks. An apolar solvent (toluene) was added as a keeper. In order to determine the optimum concentration conditions, aliquots of an acetone-solution of all active ingredients were concentrated using different combinations of time and temperature (40 °C/110 min, 45 °C/60 min, 50 °C/40 min and 55 °C/18 min). The levels of the different active ingredients after concentration were measured in the NCI mode under the conditions described for extract fraction 1. These analysis conditions were selected as they allowed the determination of ethylene dibromide (EDB) and dibromochloropropane (DBCP), the most volatile compounds covered by the method. Fig. 2 presents a comparison of the recoveries obtained under different experimental conditions for the four most volatile compounds analyzed in the NCI mode. When the concentration was performed at 40 °C, a 110 min evaporation time was required and the recoveries of EDB and DBCP were 5 and 50%, respectively. For the other pesticides analyzed, the recoveries were quantitative. Upon raising the temperature and decreasing the concentration time (55 °C/18 min) the recovery of DBCP was brought to 100% while the that of EDB was only marginally increased (10%). The recoveries of the other pesticides remained quantitative. The working conditions were therefore set to 55 °C/18 min and a specific internal standard, deuterium-labeled EDB, was added in order to compensate for the losses of EDB during concentration. Under these conditions, the corrected recovery of EDB was enhanced to 100%. For the real tobacco samples, this internal standard was

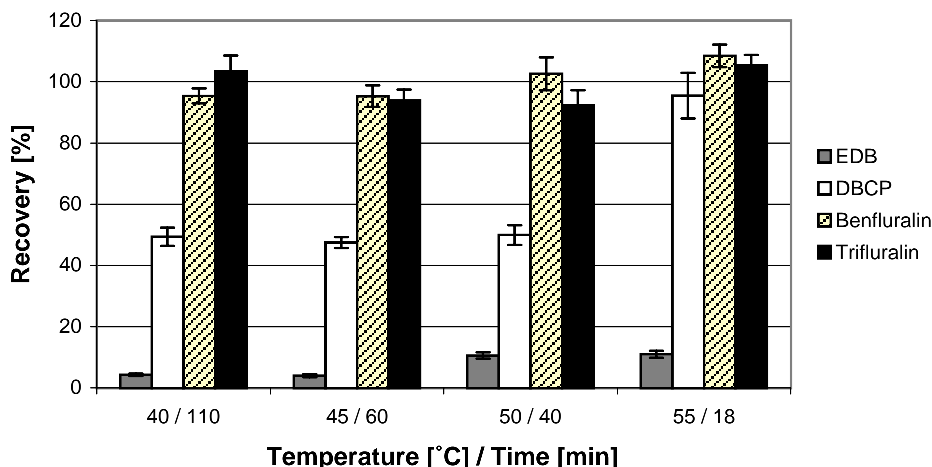

Fig. 2. Recoveries of ethylene dibromide (EDB), dibromochloropropane (DBCP), benfluralin and trifluralin obtained under different concentration conditions (time and temperature).

added to the tobacco in the PLE extraction cell, together with the other internal standards (mirex and thiofanox).

3.3. Solid-phase extraction clean-up

The SPE purification of the extract is aimed at reducing the amount of co-extractives from the tobacco matrix that otherwise would contaminate the head of the GC or LC columns. In such a case, the repeatability of the determination would be reduced and the achieved LOD would be higher. The solid-phase extraction technology offers batch processing capabilities, time savings, reduced solvent consumption, high selectivity and ease of automation. The SPE cartridges adopted in the present study (silicagel, florisil, graphitized carbon black and aminopropyl) are widely used for the analysis of the main classes of pesticide residues in food matrices [15,16].

In order to cope with the differences in the chemical properties of the considered active ingredients, three clean-up procedures were defined. Each procedure consists of two SPE clean-ups in series. This approach was necessary to achieve the clean-up efficiency required to ensure a sufficient repeatability of the determination and to avoid a loss of sensitivity of the GC–MS–MS signals (signal suppression effect). The first clean-up uses a Florisil and a silicagel cartridge and is dedicated to low-polarity compounds, i.e. organochlorine compounds and 2,6-dinitroanilines. The second clean up (silicagel followed by graphitized carbon black cartridges) is designed for compounds of intermediate and/or high polarity, i.e. acylalanines, organophosphorus and some halogen-containing pesticides. The third clean-up applies to the *N*-methylcarbamates and requires first an aminopropyl cartridge followed by a graphitized carbon black cartridge.

The performance of the SPE clean-up was assessed according to two criteria: the absence of matrix effects and a high enough recovery of the analytes to be tested. The clean-up was found to be selective; no false positives were obtained for the various major types of samples (Burley, flue cured and Oriental tobaccos as well as for selected finished products). The recovery of all active ingredients from a fortified (0.5 ppm level) PLE extract of flue-cured tobacco ranged from 85 to 100%.

3.4. GC–MS–MS analysis

The SPE-eluate containing the non-polar organochlorine compounds, 2,6-dinitroanilines and pyrethroïds as well as the eluate containing the organophosphorus compounds, the acylalanines and the polar organochlorine compounds were analyzed by GC–MS–MS using the SRM mode. In order to maximize both the sensitivity and the selectivity, different ionization modes, NCI and EI, were applied. Tables 1 and 2 show the transitions selected for each analyte.

Compounds having a high electro-affinity such as organochlorine compounds, 2,6-dinitroanilines and pyrethroïds were analyzed in the NCI mode using methane as the reagent gas. The ionization conditions for these classes of compounds are well documented in the literature. In particular, it was reported that NCI ionization with methane leads to an enhanced selectivity [17] and about 100–1000 times higher sensitivity compared to the EI or to the positive chemical ionization (PCI) modes [18,19]. The sensitivity of the MS–MS determination was evaluated by the analysis of solutions of the different compounds of interest in acetone. The sensitivity achieved in the NCI mode appeared to strongly differ among the active ingredients. Particularly low detection limits, as low as 0.025 pg injected, were observed for two 2,6-dinitroanilines, benfluralin and trifluralin while, at the other end of the observed range, the detection limit for *p*,*p*-DDT was 100 pg injected. The limited sensitivity observed for that compound was due to the low intensity of the specific transitions present in the MS spectrum. However, the achieved detection limit was still compatible with the required sensitivity for this compound and therefore, no changes in the ionization conditions were deemed necessary.

Upon analyzing fortified tobacco extracts, a lack of specificity of the available transitions was observed for three halogenated compounds (EDB, DBCP, and methoxychlor) and precluded their analysis in the NCI mode as initially planned. For these three compounds, specific transitions were identified in the EI mode that were free of interferences from the tobacco matrix. EDB, DBCP, and methoxychlor were therefore analyzed in the EI mode together with organophosphorus and acylalanine insecticides.

Twenty four analytes including acylalanines, organophosphates, ethofumesate methoxychlor, EDB

and DBCP were analyzed in the EI mode. The achieved detection limits of the MS–MS analysis were evaluated using solutions of the different compounds in acetone. The difference between the highest and the lowest detection limits was much smaller than observed for the compounds analyzed in the NCI mode. The obtained values ranged between 0.8 pg injected (dimefox) and 30 pg injected (methamidophos). Due to the low pressure at which the mass spectrometer was operated, the EI mode was easier to apply than the methane-NCI mode. In particular, the contamination of the ion source was greatly reduced, thus limiting the need for instrument maintenance.

3.5. HPLC determination of N-methylcarbamates

The *N*-methylcarbamates contained in the third SPE eluate were analyzed by HPLC with post-column reaction and fluorescence detection. This analytical approach is widely used for *N*-methylcarbamates in complex matrices as indicated in a review published by Yang et al. [20]. One paper describes the application of this analytical approach for the determination of *N*-methylcarbamate residues in tobacco [21]. The data gathered in the context of the global assessment of the method (see Tables 3–5) indicated that the quantification at three levels (0.05, 0.5 and 3 ppm) was accurate and precise. The recoveries ranged from 99 to 106% at the 3 ppm level, from 92 to 105% at the 0.5 ppm level and from 80 to 113% at the 0.05 ppm level. The relative standard deviation (R.S.D.) determined under repeatability conditions ($n = 6$) ranged between 1.1 and 7.4% at the 3 ppm level, between 1.9 and 8.1% at the 0.5 ppm level and between 3.3 and 14% at the 0.05 ppm level.

3.6. Evaluation of method performance

3.6.1. Accuracy and precision

Accuracy (recovery) and precision (repeatability) were determined at three levels (0.05, 0.5 and 3 ppm) using fortified tobacco samples. The results for six

Table 3
Recovery (%), precision (CV%), LOD, LOQ for the different active ingredients analyzed by MS/MS in the El mode

Active ingredient	Spiking level (ppm)	Recovery (%)	Precision CV(%)	Spiking level (ppm)	Recovery (%)	Precision CV(%)	Spiking level (ppm)	Recovery (%)	Precision CV(%)	LOD (ppm)	LOQ (ppm)
EDB	0.05	(*)	(*)	0.5	95	12.7	3.0	101	7.8	0.150	0.500
Dimefox	0.05	110	4.8	0.5	105	9.1	3.0	102	4.9	0.003	0.010
DBCP	0.05	108	4.1	0.5	98	7.9	3.0	95	10.0	0.010	0.040
Methamidophos	0.05	(*)	(*)	0.5	86	7.5	0.8	91	5.9	0.090	0.300
Dichlorvos	0.05	81	2.8	0.5	70	5.9	3.0	82	7.1	0.030	0.050
Mevinphos	0.05	106	4.0	0.5	104	10.5	3.0	115	5.1	0.010	0.040
Acephate	0.05	96	5.3	0.5	94	8.6	1.0	95	10.0	0.030	0.050
Dimethoate	0.05	100	4.0	0.5	92	8.6	3.0	113	4.4	0.030	0.050
Terbufos	0.05	91	4.5	0.5	89	4.7	3.0	114	6.4	0.003	0.010
Diazinon	0.05	109	6.4	0.5	101	8.1	3.0	109	5.2	0.010	0.040
Disulfoton	0.05	83	6.2	0.5	69	7.1	3.0	100	4.7	0.003	0.014
Formothion	0.05	95	3.7	0.5	115	7.0	3.0	91	9.8	0.006	0.015
Methyl-parathion	0.05	100	6.2	0.5	87	10.0	3.0	106	4.9	0.010	0.030
Metalaxyl	0.05	104	4.9	0.5	98	9.0	3.0	110	8.3	0.030	0.050
Ethofumesate	0.05	106	5.5	0.5	104	8.5	3.0	106	3.3	0.003	0.040
Malathion	0.05	101	4.8	0.5	95	10.9	3.0	102	4.1	0.010	0.040
Fenthion	0.05	95	5.6	0.5	98	10.0	3.0	97	2.5	0.030	0.050
Ethyl-parathion	0.05	112	5.4	0.5	101	7.8	3.0	105	4.8	0.006	0.020
Terbufos sulfone	0.05	102	5.4	0.5	98	7.5	3.0	110	7.1	0.010	0.030
Disulfoton sulfone	0.05	89	6.5	0.5	73	7.7	3.0	109	5.0	0.010	0.030
Fenamiphos	0.05	106	4.8	0.5	126	5.2	3.0	107	2.9	0.020	0.050
Benalaxyl	0.05	108	2.5	0.5	115	6.8	3.0	108	4.5	0.030	0.050
Methoxychlor	0.05	109	5.9	0.5	128	4.7	3.0	95	9.6	0.010	0.030
Phosalone	0.05	99	5.7	0.5	120	6.7	3.0	103	5.4	0.010	0.040

(*) Data not available, fortification level below LOQ.

Table 4
Recovery (%), precision (CV%), LOD, LOQ for the different active ingredients analyzed by MS/MS in the NCI mode

Active ingredient	Spiking level (ppm)	Recovery (%)	Precision ($n = 6$) CV(%)	Spiking level (ppm)	Recovery (%)	Precision ($n = 6$) CV(%)	Spiking level (ppm)	Recovery (%)	Precision ($n = 6$) CV(%)	LOD (ppm)	LOQ (ppm)
Trifluralin	0.04	84	7.7	0.420	98	5.2	2.0	111	5.8	0.001	0.003
Benfluralin	0.05	84	7.7	0.500	95	4.8	2.0	100	7.3	0.001	0.003
α-HCH	0.06	96	7.2	0.585	99	6.1	3.0	109	3.8	0.020	0.060
HCB	0.06	63	7.2	0.590	74	4.5	3.0	109	3.8	0.010	0.030
Dichloran	0.05	91	5.1	0.518	94	6.6	3.0	91	5.3	0.018	0.050
β-HCH	0.07	99	4.4	0.570	103	9.0	3.0	100	5.5	0.025	0.070
Lindane	0.05	97	6.1	0.520	103	3.2	3.0	116	4.8	0.010	0.030
d-HCH	0.05	95	4.8	0.503	104	6.2	3.0	100	7.3	0.020	0.050
Heptachlor	0.05	87	2.9	0.500	102	3.8	3.0	123	6.0	0.020	0.050
Aldrin	0.05	71	5.3	0.525	85	2.8	3.0	117	6.3	0.010	0.030
Butralin	0.05	82	8	0.548	95	5.5	3.0	98	7.2	0.001	0.005
Isopropalin	0.04	79	6.7	0.445	103	9.4	3.0	98	6.4	0.001	0.003
Pendimethalin	0.04	86	7.1	0.428	105	6.6	3.0	99	8.1	0.001	0.003
Heptachlor_epoxide	0.04	85	6.9	0.415	100	4.6	3.0	102	3.4	0.010	0.040
trans-Chlordane	0.05	97	4.5	0.500	101	11.1	0.5	110	5.2	0.002	0.006
o,p-DDE	0.06	87	4.1	0.558	101	9.6	3.0	101	3.5	0.010	0.030
α-Endosulfan	0.06	101	5.2	0.618	93	5.5	3.0	103	3.5	0.010	0.040
Flumetralin	0.05	85	6.6	0.455	100	6.8	3.0	111	7.8	0.001	0.003
p,p-DDE	0.05	77	4.4	0.543	93	3.0	3.0	97	4.3	0.005	0.015
Dieldrin	0.06	90	4.8	0.593	90	3.1	3.0	107	4.1	0.010	0.030
o,p-TDE	0.06	100	4.4	0.578	114	6.7	3.0	110	4.2	0.020	0.060
Nitrofen	0.05	89	5.3	0.478	92	9.5	3.0	100	3.5	0.009	0.010
Endrin	0.05	88	6.1	0.533	90	5.3	3.0	101	5.8	0.014	0.030
β-Endosulfan	0.06	108	4.8	0.560	97	3.3	3.0	87	3.3	0.010	0.030
p,p-TDE	0.06	(*)	(*)	0.603	118	18.4	3.0	101	7.8	0.060	0.230
o,p-DDT	0.06	87	3.4	0.583	105	14.4	3.0	96	11.1	0.030	0.060
Endosulfan_sulfate	0.05	97	3.5	0.470	106	4.3	3.0	97	5.8	0.010	0.040
p,p-DDT	0.09	94	5.3	0.875	99	15.4	3.0	98	5.6	0.030	0.080
Camphechlor[a]	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.001	0.004
Lambda-Cyhalothrin (1+2)[b]	n.a.	n.a.	n.a.	0.500	94	2.2	3.0	86	4.6	0.003	0.010
cis-Permethrin (1)	0.05	81	12	0.500	104	17.8	3.0	98	7.1	0.020	0.050
trans-Permethrin	0.07	91	10.6	0.687	105	15.4	0.5	87	5.6	0.030	0.060
1-Cypermethrin (β)	0.05	96	9.8	0.543	119	10.3	1.3	98	7.8	0.010	0.030
2-Cypermethrin	0.04	92	12.4	0.414	109	13.2	1.1	107	9.2	0.010	0.030
3-Cypermethrin (α)	0.04	75	8.7	0.436	113	9.5	2.0	101	7.2	0.010	0.030
4-Cypermethrin (τ)	0.06	92	10.1	0.623	110	11.6	1.6	97	6.3	0.020	0.060
RS/SR-Fenvalerate	n.a.	n.a.	n.a.	0.333	83	4.3	1.8	108	10.4	0.010	0.060
SS/RR-Fenvalerate	n.a.	n.a.	n.a.	0.167	113	9.2	1.2	103	9.3	0.010	0.030
Deltamethrin (*cis* + *trans*)	0.06	88	11.5	0.643	95	16.7	3.0	106	4.1	0.010	0.030

n.a.= not analyzed; (*) data not available, fortification level below LOQ.

[a] Camphechlor is a mixture of a high number of chlorinated camphenes. No quantitative analysis was performed. The above LOD and LOQ figures for camphechlor are indicative; they refere to the analysis of the most abundant compound in the camphechlor standard.

[b] The SRM chromatogram of λ-cyhalothrin consists of two signals corresponding to difference isomers. The cyhalothrin residues were calculated as the sum of both isomers.

Table 5
Recovery (%), precision (CV%), LOD, LOQ for the *N*-methylcarbamates

Active ingredient	Spiking level (ppm)	Recovery (%)	Precision CV(%)	Spiking level (ppm)	Recovery (%)	Precision (n=6) CV(%)	Spiking level (ppm)	Recovery (%)	Precision CV(%)	LOD (ppm)	LOQ (ppm)
Aldicarb sulfoxide	0.05	92	5.9	0.500	79	7.0	3.0	99	2.7	0.015	0.050
Aldicarb sulfone	0.05	99	1.9	0.500	100	3.3	3.0	102	1.8	0.010	0.035
Oxamyl	0.05	101	2.3	0.500	87	8.6	3.0	104	2.5	0.015	0.050
Methomyl	0.03	101	5.2	0.300	74	14.0	3.0	99	7.4	0.010	0.030
Aldicarb	0.05	86	3.3	0.500	84	13.4	3.0	105	2.8	0.015	0.050
Propoxur	0.05	99	2.9	0.500	88	9.8	3.0	105	1.1	0.015	0.050
Carbofuran	0.05	100	3.9	0.500	99	9.4	3.0	105	1.3	0.015	0.050
Carbaryl	0.05	105	8.1	0.500	113	7.1	3.0	100	6.1	0.010	0.035
Ethiofencarb	0.05	98	7.4	0.500	85	6.3	3.0	105	1.8	0.015	0.050
Methiocarb	0.05	107	8.9	0.500	98	5.6	3.0	99	5.8	0.030	0.050

replicate analyses are reported in Tables 3 and 4. At the 3 ppm spiking level, recoveries ranged from 82% (dichlorvos) to 115% (mevinphos) with the exception of heptachlor (123%). At the 0.5 ppm level, recoveries ranged from 70% (dichlorvos) to 128% (methoxychlor) while at the 0.05 ppm level, they ranged from 71% (aldrin) to 110% (dimefox) with one exception, HCB at 63%.

The achieved precision expressed as the repeatability R.S.D. ranged from 1.1% (carbofuran) to 11.1% (*o*,*p*-DDT) at the 3 ppm level and from 1.9% (aldicarb sulfone) to 11.5% (deltamethrin) at the 0.5-ppm level while at the 0.05-ppm level, they ranged from 2.8% (aldrin) to 18.4% (*p*,*p*-DDD).

3.6.2. Selectivity

The selectivity of the GC–SRM–MS–MS method was verified by processing and analyzing samples that tested negative for the targeted pesticides according to the current test procedure. These samples included flue-cured, burley and Oriental tobaccos, as well as finished products. No interferences were noticed close to the retention time of the analytes. The adopted chromatographic conditions allowed to separate compounds which had to be detected using the same SRM transitions, e.g. some compounds of the DDT family and isomers of permethrin and of cypermethrin. Two 2,6-dinitroanilines, trifluralin and benfluralin, differing only slightly in the substitution of the amino function (*N*,*N*-dipropyl versus *N*-butyl-*N*-ethyl, respectively) proved particularly difficult to differentiate. The available specific SRM transitions were the same for both compounds and the retention times were very close ($t_R = 12.53$ min for benfluralin and $t_R = 12.61$ min for trifluralin). However, these retention times were shown to remain perfectly stable during the analysis of a batch of samples, so that the achieved GC separation proved sufficient to ensure a correct identification of the trifluralin and the benfluralin GC signals. This fact was confirmed by the analysis of tobacco extracts fortified with either trifluralin or benfluralin. Based on the analysis of 40 such samples, performed on different analysis days, no case of misidentification was observed. The method allows therefore differentiating between those two compounds. Fig. 3 presents the SRM chromatogram of a tobacco sample fortified at a level of 0.3 ppm with both compounds.

3.6.3. Limits of detection (LOD) and of quantification (LOQ)

The LOD and LOQ were determined as the residue levels, in ppm (μg/g of tobacco), leading to signal/noise ratios of 3 and 10, respectively. The LOD of the compounds analyzed by tandem mass spectrometry were comparable for both ionization modes, EI and NCI, with most values ranging between 0.001 and 0.01 ppm. Two compounds were clearly out of that range: EDB with a LOD of 0.15 ppm and *p*,*p*-DDD with a LOD of 0.06 ppm. In both cases, the reason of the elevated LOD was the low intensity of the existing MS–MS transitions. In addition, for EDB, the low recovery achieved during the vortex concentration also contributed to the limited sensitivity.

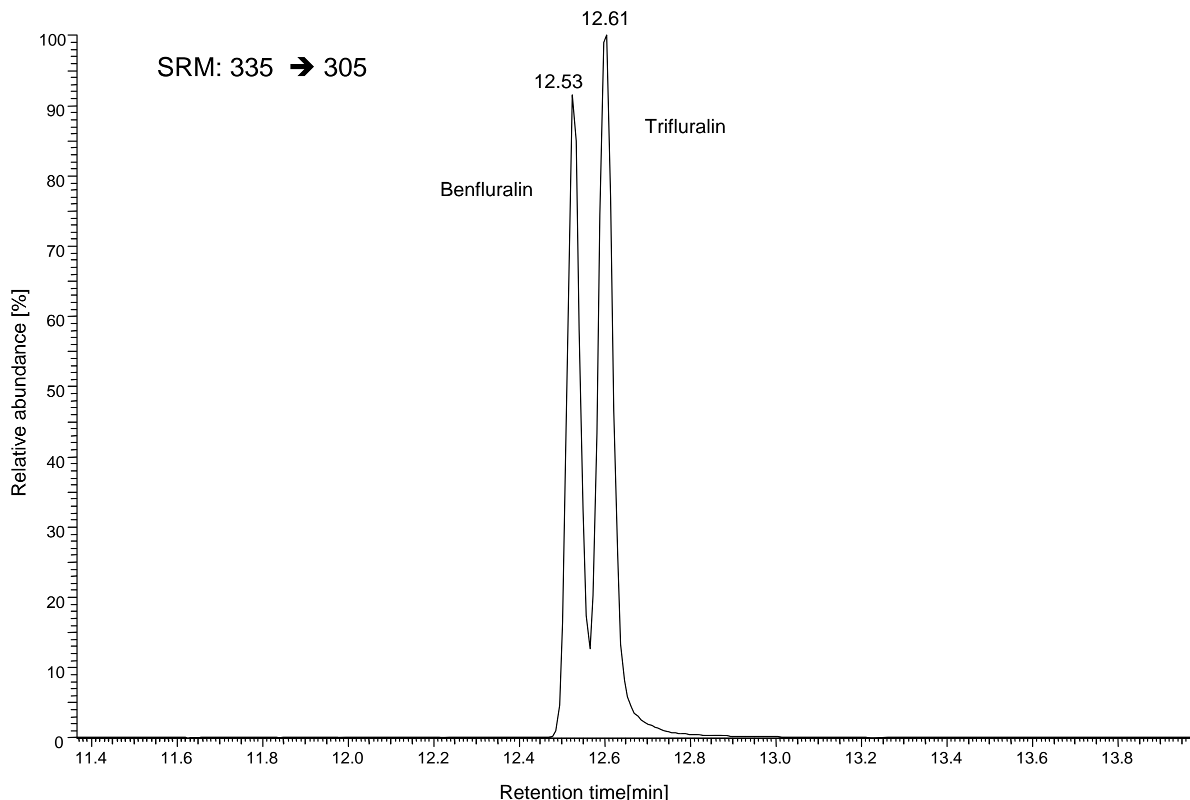

Fig. 3. SRM chromatogram of a tobacco sample fortified with 0.3 ppm of benfluralin and trifluralin.

In line with the observed distribution of the LOD, most of the LOQ were comprised between 0.003 and 0.03 ppm with clearly higher values for EDB (0.5 ppm) and *p*,*p*-DDD (0.2 ppm). These values were, however, still in line with the required sensitivity for those compounds. The difference in sensitivity achieved for EDB, *p*,*p*-DDD and the other analytes represented by endosulfan sulfate is shown in Fig. 4. Endosulfan sulfate was selected as a benchmark as its LOD is situated in the middle of the observed range.

3.6.4. Sample throughput and coverage

The new multiresidue method provides a potential for the analysis of up to 100 samples per week on an on-going basis. Compared to the method previously used in our laboratory, this represents a five- to seven-fold increase without additional resources in terms of laboratory personnel. It is important to underline that the MS–MS technology is a major contributor to this achievement. The high selectivity achieved by tandem mass spectrometry in the SRM mode dramatically decreases the chemical noise of the chromatographic method and places much less stringent requirements on the separation of the peaks. This in turn makes it possible to apply relatively simple clean up procedures and to shorten the chromatographic analysis. Three labor-intensive stages (liquid–liquid partition, gel permeation chromatography and SPE) were replaced by an automated SPE procedure. The number of separate chromatographic analyses could be reduced from five to three and the duration of the GC runs was shortened by a factor of two. An additional benefit of the lower constraints on the quality of the achieved clean-up and GC separation is the possibility of extending the coverage of the method to additional active ingredients with, in most cases, no significant method development.

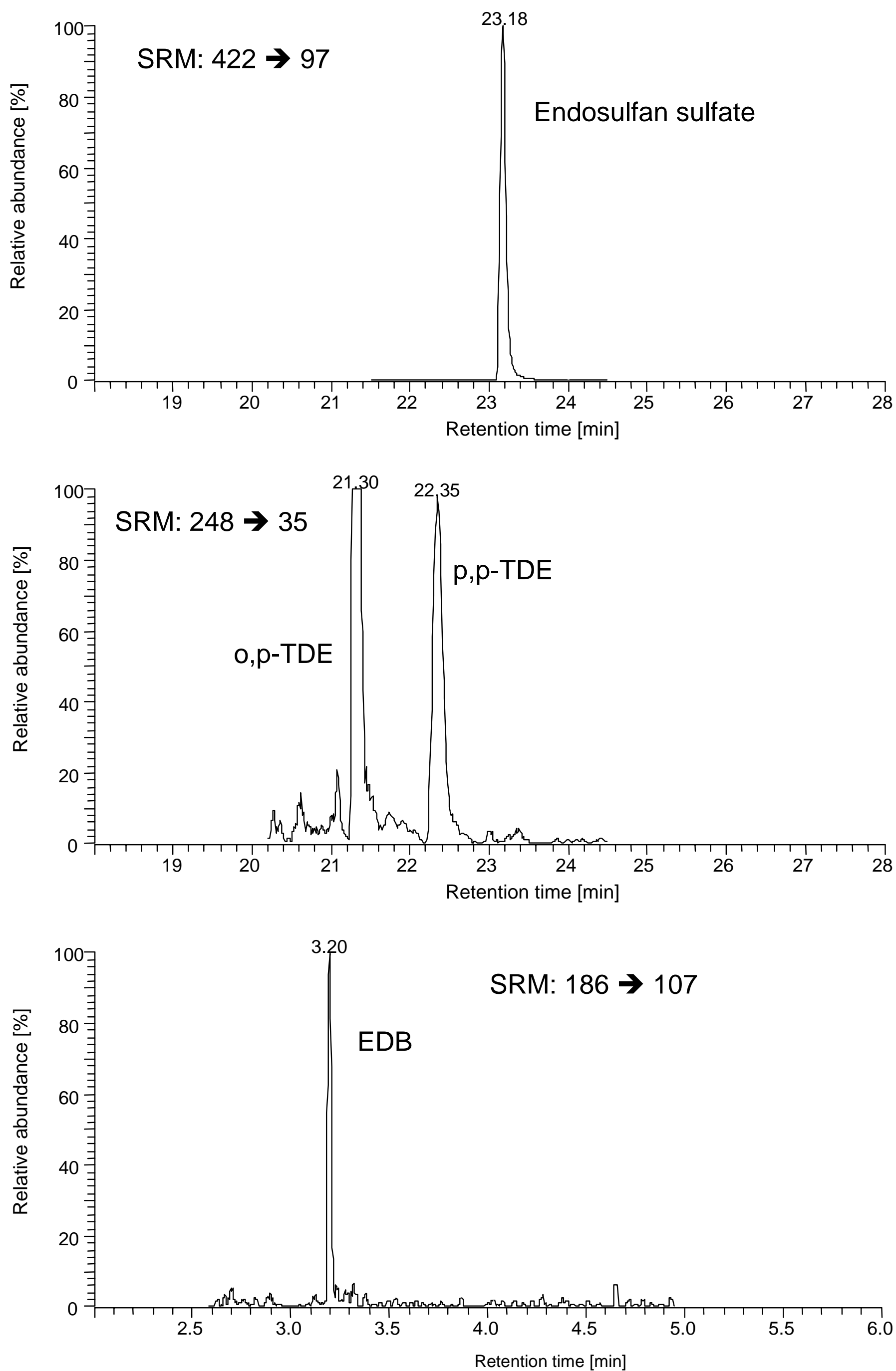

Fig. 4. SRM chromatograms of endosulfan sulfate, *p*,*p*-DDD (*p*,*p*-TDE) and EDB obtained upon analyzing a tobacco sample fortified with 0.5 ppm of each active ingredient.

The weekly analysis capacity mentioned above is close to the maximum that can be achieved for the determination of the *N*-methylcarbamates. Therefore, a further upgrade of the new method will be the substitution of the HPLC–fluorescence analysis by HPLC coupled to tandem mass spectrometry (HPLC–MS–MS). This technology will allow one to process more samples by dramatically reducing the chromatographic run time and enable the extension of the coverage to a wider range of carbamate compounds.

4. Conclusion

A new multiresidue method covering 74 active ingredients was developed. The application of recent analytical technologies provided a potential for a dramatic increase of the sample throughput compared to the conventional methods. The key element for this was the application of tandem mass spectrometry. Due to the specificity of this technique, the sample preparation could be simplified and automated and the GC runs could be made shorter, while providing an extremely high level of confidence in the identity of the detected pesticides.

The performance of the method in terms of selectivity, accuracy, precision and limits of detection/quantification was in line with requirements for all considered 74 active ingredients.

The good performance achieved by the method and the possibility to easily automate the time consuming clean-up stage make this method well suited for routine analyses in situations where a large number of samples need to be processed with a limited laboratory staff.

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